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Gene expression profiling in the human alcoholic brain

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Abstract

Long-term alcohol use causes widespread changes in gene expression in the human brain. Aberrant gene expression changes likely contribute to the progression from occasional alcohol use to alcohol use disorder (including alcohol dependence). Transcriptome studies have identified individual gene candidates that are linked to alcohol-dependence phenotypes. The use of bioinformatics techniques to examine expression datasets has provided novel systems-level approaches to transcriptome profiling in human postmortem brain. These analytical advances, along with recent developments in next-generation sequencing technology, have been instrumental in detecting both known and novel coding and non-coding RNAs, alternative splicing events, and cell-type specific changes that may contribute to alcohol-related pathologies. This review offers an integrated perspective on alcohol-responsive transcriptional changes in the human brain underlying the regulatory gene networks that contribute to alcohol dependence.

Keywords

gene expression; co-expression networks; transcriptional regulation; RNA-seq; alcohol

1. Introduction

Alcohol use disorder (AUD) involves a progression from impulsive to compulsive drinking behavior resulting in long-term, relapsing alcohol use. As individuals transition across these drinking domains, a negative emotional state (i.e. the “dark side of addiction”) emerges upon withdrawal, resulting in preoccupation with obtaining alcohol (Koob and Le Moal, 2005). Alcohol dependence can be conceptualized as a three-stage cycle, *binge/intoxication*, *withdrawal/negative affect*, and *preoccupation/craving*—that progressively escalates over time due to compensatory allostatic changes in the brain’s stress and reward systems. These aberrant brain functions are likely a consequence of altered gene and protein expression that

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underlie persistent plasticity changes in the neural circuits responsible for different motivational systems.

In individuals escalating to alcohol dependence, there is a complex interplay among predisposition genes (genes associated with risk), environmental factors, and history of alcohol exposure (Contet, 2012). These combined factors likely contribute to system-wide epigenetic alterations, post-translational modifications, and long-term allostatic changes in brain regions that underlie AUD (e.g. mesolimbic, extended amygdala, frontal cortex) (Contet, 2012). Recent progress in understanding genes and gene networks associated with AUD in brain has come from animal studies of excessive alcohol consumption and human alcoholics.

Since the introduction of microarray technology, transcriptome profiling of human postmortem brain tissue has been used to study the genomics of alcohol dependence (reviewed in (Farris & Mayfield, 2014; Mayfield, Harris, & Schuckit, 2008)). The functional categories of over-represented, differentially expressed genes that have been identified suggest that the transcriptional effects of long-term alcohol consumption contribute to neurotoxicity and changes in neuroplasticity. However, the biological significance of the individually expressed genes is not apparent in genome-wide transcriptome studies. Transcriptomes from complex tissues, such as human brain, may be organized into networks of co-expressed genes that are thought to better reflect the biological functions and organization of the tissue (Farris et al., 2015a; Oldham et al., 2008; Ponomarev et al., 2010).

Recent developments in bioinformatics and sequencing techniques have allowed analyses to shift from individual genes to networks of co-expressed genes, thus providing a more integrated, biologically relevant view of AUD. Application of novel bioinformatics techniques, such as weighted gene co-expression network analysis (WGCNA), has uncovered gene co-expression networks associated with alcohol dependence (Ponomarev et al., 2012). Furthermore, in the last five years, RNA-seq has become an invaluable tool to analyze the transcriptome. Due to advances in sequencing technology, RNA-seq makes it possible to identify exons and introns by the boundaries of their genes, to identify transcription initiation start sites and splicing variants, and to quantify exon and splicing isoform expression (Farris and Mayfield, 2014). This allows the study of alcohol-induced alterations in gene expression in the human brain at an unprecedented level, and the advancement of potentially relevant treatment strategies for normalizing addiction-related changes in gene expression. With these rapid advances also come challenges that are further amplified when considering the broad range of ‘omic’ approaches and modeling requirements for understanding complex disease (Coveney et al., 2016).

One particular challenge of the post-genomic “big data” era is to determine the degree to which alterations in gene expression networks and gene structure can be used as predictive models that infer causal relationships associated with AUD. For example, there is accumulating genomic evidence for alcohol-induced inflammatory-and immune-related signaling from genetic association studies in alcoholics (Edenberg et al., 2008; Pastor et al., 2000; Pastor et al., 2005; Saiz et al., 2009), gene expression microarray studies in postmortem brains of alcoholics (Liu et al., 2006; Ökvist et al., 2007), and transcriptome

meta-analyses in selectively bred (Mulligan et al., 2006) and ethanol-exposed mice (Gorini et al., 2013a; Gorini et al., 2013b; Nunez et al., 2013; Osterndorff-Kahanek et al., 2013). Network analyses have identified highly connected hub genes that are related to inflammatory/immune responses and mediate their effects through NF- κ B. These studies implicated inhibitor of nuclear factor kappa-B kinase subunit beta (IKK β) as having a modulatory role in alcohol drinking. In a proof-of-concept study, Truitt and colleagues showed that pharmacological and genetic inhibition of IKK β decreased voluntary ethanol consumption, providing initial support for IKK β as a potential therapeutic target for alcohol abuse (Truitt et al., 2016), as well as functional evidence that gene network analysis is a viable approach to identify new treatments.

In the following sections, we describe the factors that influence the reliability and consistency of gene expression data obtained from human postmortem brain and review the recent brain transcriptome studies from alcoholic individuals (see Table 1). We highlight how advances in sequencing technology and data analysis have revealed novel transcriptional regulation in the alcoholic human brain, summarize the functional categories and pathways that are associated with long-term alcohol abuse, and discuss advances in cell-type specific profiling studies that are refining our understanding of AUD.

2. Human postmortem tissue quality control

Human donor tissue is limited, and there are often difficulties in establishing proper control groups. For accurate transcriptome analysis in postmortem tissue, it is essential to obtain high quality from established brain banks such as the New South Wales Tissue Resource Centre at The University of Sydney, Australia (Sutherland et al., 2014; Sutherland et al., 2016). There are several antemortem and postmortem factors that can degrade RNA quality and potentially affect the transcriptome profile of interest (Harrison et al., 1995; Lipska et al., 2006; Stan et al., 2006; Tomita et al., 2004). These variables include, but are not limited to the following: postmortem interval, tissue pH, agonal state, and tissue origin. The factors affecting RNA quality and transcript levels in postmortem brain samples have been intensively studied (Harrison et al., 1995; Lipska et al., 2006; Stan et al., 2006; Tomita et al., 2004). Yet, the interplay of RNA abundance, transcript expression level, etc., is still not well understood, and the variables that are most predictive of RNA quality and transcript abundance are highly contested.

Postmortem interval, agonal state, and tissue pH have all been found to weakly correlate with RNA quality (Barton et al., 1993; Harrison et al., 1995; Johnston et al., 1997; Li et al., 2004; Tomita et al., 2004). In addition to reducing RNA quality, prolonged agonal states and low tissue pH can lead to differences in the representation of certain transcripts (Harish et al., 2013; Li et al., 2004; Lipska et al., 2006). For instance, patients who suffered from prolonged agonal states had lower tissue pH and decreased expression of specific genes, especially those involved in energy metabolism and proteolytic activities (Harish et al., 2013; Li et al., 2004). Increased expression of genes encoding stress-response proteins and transcription factors has also been observed in patients with prolonged agonal states and low pH (Li et al., 2004). The correlation between changes in expression and these variables thus appears to be transcript specific rather than an overall degradation of RNA (Harish et al.,

2013). Several precautions should be taken to reduce confounding factors that impact RNA quality. For example, it has been suggested that tissue with a pH < 6.5 should be excluded from microarray analysis (Li et al., 2004). Long postmortem intervals and severe agonal states (e.g. brain trauma, stroke) should also be excluded, and histopathological criteria should be used to provide evidence that the agonal state conforms to accepted guidelines.

Besides general quality control measures, additional factors must be considered when designing experiments to profile human postmortem brain. First, alcoholic individuals with a history of polydrug abuse should be excluded or appropriately controlled. Polydrug use can be considered an exclusion factor but this may depend on the population cohort. Although challenging, one could control for polydrug use by using appropriate control groups or by using statistical covariates. It should be noted that there is a high incidence of smoking among alcoholics and nicotine use represents an important covariate to investigate. Gene expression differences between control subjects and alcoholics can thus potentially be driven by nicotine dependence or the interaction between nicotine and alcohol. Second, cases with concomitant diseases such as cirrhosis of the liver, Wernicke-Korsakoff syndrome, or hepatic encephalopathies should also be considered in the experimental design. As with polydrug use, exclusion of concomitant diseases is not necessarily a requirement for all studies. Concomitant diseases as an experimental factor in study designs can be useful in creating more homogenous groups and reducing heterogeneity (Liu et al., 2004; Liu et al., 2006). Moreover, alcoholics with cirrhosis or other concomitant diseases can represent a more severe subtype and thus enhance the effect size (Liu et al., 2007). For example, comparison of brain gene expression from alcoholics with cirrhosis and those without revealed widespread differences in transcriptome patterns in cirrhotic versus noncirrhotic alcoholics and these differences in gene expression likely contribute to the more severe brain dysfunction observed in cirrhotic alcoholics (Liu et al., 2007). Blood alcohol concentration at the time of death is not currently available for all cases but is an important factor when subdividing cases on the basis of drinking behavior. A record of blood alcohol concentration in human subjects may be relevant considering that alcohol-responsive gene networks depend upon the duration of ethanol consumption in mouse models of excessive drinking (Osterndorff-Kahanek et al., 2015). Furthermore, inclusion criteria should account for variables such as age, ethnicity, developmental disorders, history of other psychiatric or neurological disorders, and other degenerative diseases. Minimizing confounding variables is essential to uncover the biologically relevant alcohol-responsive gene networks in humans that may underlie the specific pathophysiology of AUD.

3. Biological co-expression networks: Transcriptional regulation in alcohol use disorder

Although many genes are differentially expressed in the brains of alcoholic subjects, the observed fold-changes in individual genes are relatively small. Overall expression differences between alcohol-dependent cases and matched healthy controls often range from 20–50% (Liu et al., 2006). This is consistent with genome-wide association studies (GWAS) showing that multiple genes influence the risk for AUD, while individual genes contribute a relatively small effect (e.g., SNP odds ratios are typically <1.5 with individual SNP

heritability of ~1–2%) (Agrawal and Bierut, 2012; Edenberg and Foroud, 2006; Gelernter et al., 2014). With the exception of some genes involved in alcohol metabolism (Zhang et al., 2014a) that we discuss later in this review, individual genes are not considered to be strong predictors of AUD susceptibility nor do they contribute to the majority of AUD pathophysiology. It is hypothesized that alcohol dependence is shaped, in part, by persistent alterations in networks of co-expressed genes that collectively mediate excessive drinking and other alcohol-dependent phenotypes (Farris et al., 2015b; Farris and Mayfield, 2014).

WGCNA of human brain tissue has been valuable in the identification of cell-type specific networks that are shared among distinct brain regions and in assigning cell-type classification to predicted protein function (Oldham et al., 2008). Oldham and colleagues demonstrated the ability of a network approach to provide discrete insight into the brain transcriptome. Similar network-based approaches have been used to identify gene co-expression modules that are associated with other psychiatric disorders, such as bipolar disorders and schizophrenia (Chen et al., 2013; Torkamani et al., 2010), as well as alcohol and other drug of abuse disorders (Farris et al., 2015a; Ponomarev et al., 2012; Zhang et al., 2014a). Although co-expression networks for alcohol-related phenotypes are still emerging, it is likely that RNA-seq studies will offer greater insight into drug-induced transcriptional changes (Farris and Mayfield, 2014). For instance, RNA-seq profiling of human postmortem prefrontal cortex revealed that gene network structure was significantly correlated with lifetime alcohol consumption in addition to an overall loss in network structure (Farris et al., 2015a; Farris and Mayfield, 2014). Intermodular correlations were found to be significantly higher among modules associated with alcohol drinking compared to modules with low connectivity, implying integrated biological function for a set of gene modules directly related to lifetime alcohol consumption in humans. The differences in intermodular connectivity suggest that the neurobiology of alcohol dependence may be due to altered covariation of gene modules, rather than discrete changes in differentially expressed genes across the transcriptome (Farris and Mayfield, 2014).

3.1: Epigenetic modifications in the human alcoholic brain

Bleich and Hillemacher (2009) initially demonstrated that alcohol alters DNA methylation and expression of individual genes. The first integrative view of transcriptome alterations in human alcoholic brain was performed in the superior frontal cortex, basolateral amygdala, and central amygdala using a microarray platform and WGCNA (Ponomarev et al., 2012). This study identified an unprecedented role for epigenetic regulation in alcoholics. Most genes from GC-rich modules were up-regulated in alcohol abusers, while genes from GC-poor modules were mainly down-regulated. The importance of the genomic GC content in regulating gene expression is well established because many transcription factors and other parts of chromatin modification complexes preferentially bind to GC- or AT-rich motifs (Dekker, 2007; Zhang et al., 2004). Up-regulation of GC-rich modules suggests a critical role of chromatin modifications in the modulation of gene expression in the alcoholic brain (Ponomarev et al., 2012). In addition, alcohol-induced up-regulation of genes in GC-rich modules is hypothesized to be associated with increased levels of H3K4 trimethylation (H3K4me3) in promoter regions, again suggesting coordinated regulation of chromatin modification complexes. Furthermore, a global down-regulation of DNA methyltransferase

(*DNMT1*) was observed in all three brain regions of alcoholics, indicating a system-wide change in epigenetic regulation of gene expression. The down-regulation of *DNMT1* is consistent with literature showing hypomethylation states in alcoholic brain and blood as well as in cancer and neurodegenerative disorders (Clark et al., 2015; Hervouet et al., 2010; Wang et al., 2016; Zhang et al., 2014a). Recent studies have even suggested that alterations in DNA methylation in the prefrontal cortex are associated with (and might result in) increased risk for AUD (Wang et al., 2016).

System-wide epigenetic modifications have also been observed in the hippocampus of human alcoholics and chronic cocaine users (Farris et al., 2015a; Zhou et al., 2011). Chronic alcohol or cocaine exposure altered the expression of genes that are responsible for the regulation of transcription, gene silencing, and chromatin modifications (Zhou et al., 2011). In alcoholic cases, there were H3K4me3 signal changes at the promoters of > 700 expressed genes (uncorrected $P < 0.05$); however, there was not a significant correlation or individual locus overlap between H3K4me3 and gene expression changes, suggesting that alcohol exposure has only a modest effect on histone H3K4me3 in the hippocampus of human alcoholics. A recent study expanded upon the role of chromatin modifications in regulating gene expression by comparing gene modules constructed from ChIP-seq and RNA-seq datasets obtained from the hippocampus of human alcoholics and chronic cocaine users (Farris et al., 2015a). In contrast to the modest effects observed previously (Zhou et al., 2011), Farris and colleagues observed 35 significant overlaps between the two datasets with 83% of modules having a significant positive correlation between H3K4me3 and transcript abundance. This provided evidence for coordinated regulation of several gene sets in parallel to H3K4me3 modification, suggesting a potential causative relationship between these events. Comparison of these results with previously published gene networks in alcoholic superior frontal cortex (Ponomarev et al., 2012) showed that alcohol-regulated modules overlapped across brain regions, with the majority of genes in these modules being regulated in the same direction in both studies. Collectively, transcriptomic studies suggest that chronic alcohol consumption or cocaine use alters regulation of transcription via system-wide epigenetic modifications in brain.

In addition to altered DNA methylation, histone modifications are also prevalent in human alcoholic brain (Ponomarev et al., 2012). Moreover, these changes are observed in other species. For example, binge consumption of alcohol in humans and daily operant alcohol self-administration in rats both increased *HDAC* expression in peripheral blood (Lopez-Moreno et al., 2015). In rodent models, several studies reported ethanol-induced epigenetic alterations in H3 acetylation and *HDAC* expression (Pandey et al., 2008; Pascual et al., 2012; Starkman et al., 2012; Warnault et al., 2013). Ethanol-induced changes in *HDAC* expression have also been observed in neuronal cell culture models (Agudelo et al., 2011; Agudelo et al., 2012). HDAC inhibitors are effective in countering ethanol-induced behaviors and epigenetic changes in *HDAC*. For example, trichostatin A (TSA) treatment reversed ethanol-induced tolerance, drinking, and anxiety by inhibiting HDAC activity in the amygdala of rats (Alaux-Cantin et al., 2013; Sakharkar et al., 2014). Treatment with the HDAC inhibitor sodium butyrate blocked both the acquisition and the expression of ethanol-induced behavioral sensitization in mice (Legastelois et al., 2013). Furthermore, systemic treatment of HDAC class I and II inhibitors, TSA and SAHA (suberanilohydroxamic acid),

and the more selective MS-275 (a histone deacetylase inhibitor), decreased binge-like ethanol consumption in mice (Warnault et al., 2013). Intracerebral injection of MS-275 also increased acetylation of HDAC4 in the nucleus accumbens and striatum of dependent rats, which reduced ethanol self-administration, suggesting that it produced a direct central nervous system (CNS) effect (Jeanblanc et al., 2015). HDAC inhibitors can also reverse ethanol-induced changes in neuronal transmission; for example, treatment with TSA or SAHA reversed GABA hyposensitivity of ventral tegmental area dopaminergic neurons in mice during withdrawal (Arora et al., 2013). In a clinical study, the HDAC inhibitor, valproate, attenuated withdrawal symptoms and relapse in human alcoholics (Longo et al., 2002; Myrick et al., 2000). These data provide evidence that certain HDAC inhibitors may have therapeutic efficacy by reversing alcohol-induced epigenetic modifications and normalizing aberrant gene expression changes after chronic alcohol exposure.

The findings outlined above suggest that epigenetic alterations within addiction-related brain circuits in the frontal cortex and hippocampus could perturb the regulation of cognitive and emotional processes, resulting in an overvaluing of drug reinforcers at the expense of undervaluing natural rewards (Koob and Volkow, 2010, 2016; Volkow et al., 2016). Moreover, changes in epigenetic regulation in the hippocampus could produce widespread gene expression changes that lead to aberrant neuroadaptations in conditioned learning and declarative memory (White, 1996). Gene expression alterations in the extended amygdala circuit, including the basolateral and central nucleus of the amygdala, could contribute to anxiety/aversive-like effects of acute withdrawal and increased drug intake associated with dependence (Koob and Volkow, 2010; Volkow et al., 2016). Thus, alcohol-induced epigenetic regulation has the potential to impact different stages of the addiction cycle (Fig. 1). Moreover, some HDAC inhibitors, such as SAHA, are already approved for treatment of primary cutaneous T-cell lymphoma and other types of cancer while other HDAC inhibitors are in clinical trials for neuropsychiatric disorders (Falkenberg and Johnstone, 2014). Therefore, HDAC inhibitors represent a potential class of drug that could be repurposed for the treatment of AUD.

3.2: MicroRNAs as transcriptional regulators

Protein-coding genes have traditionally been the most well-studied sequences in the human genome; however, these genes account for less than 2% of known structural and regulatory molecular elements (Alexander et al., 2010). Non-coding RNAs (ncRNAs) are key transcriptional and post-translational regulators, representing a large and diverse class of epigenetic factors that coordinate normal patterns of gene expression (Alexander et al., 2010). In recent years, it has become increasingly clear that the non-protein-coding portion of the genome is functionally important and required for normal development and physiology, and is also linked with a number of diseases (Cech and Steitz, 2014; Mercer et al., 2009). This fundamental change in our understanding has resulted from data generated by advanced sequencing techniques that have transformed our view of the central dogma that DNA is transcribed into RNA which is translated into protein. ncRNAs make up a large portion of the genome (Carninci et al., 2005; Consortium, 2012; Farris et al., 2015a), and distinct ncRNA regulatory mechanisms in brain are thought to influence the development and progression of psychiatric disorders (Kocerha et al., 2015; Sartor et al., 2012), including

alcoholism (Farris and Mayfield, 2014). MicroRNAs (miRNAs; ~17–24 nucleotides) are one class of ncRNAs that are known to regulate the expression of many genes by binding to miRNA-regulation elements in their transcriptional targets. This direct targeting can result in suppression of translation, degradation of the targeted mRNA transcript, or even increased mRNA translation, resulting in enhanced expression of target genes (Breving and Esquela-Kerscher, 2010; Filipowicz et al., 2008; Vasudevan et al., 2007). miRNAs have important roles in multiple biological processes including neuronal differentiation, brain development, synapse formation and plasticity, and neurodegeneration (Fiorenza and Barco, 2016). In addition, miRNAs have roles in aberrant biological processes that underlie neurodegenerative diseases (e.g., Alzheimer's and Parkinson's disease) and neuropsychiatric illnesses (e.g., schizophrenia, bipolar disorder), as well as the cellular adaptations induced by drugs of abuse (e.g., nicotine, cocaine, opioids, alcohol) (Miranda et al., 2010; Nunez and Mayfield, 2012).

miRNAs were significantly up-regulated in the frontal cortex of human alcoholics (Lewohl et al., 2011; Manzardo et al., 2014; Nunez and Mayfield, 2012), and may have roles in apoptosis, cell cycle regulation, cell adhesion, oligodendrocyte proliferation, synaptic transmission, ion channel function, and immune signaling based on functional classification of predicted target genes (Nunez and Mayfield, 2012). Predicted target mRNAs of up-regulated miRNAs were significantly over-represented among alcohol-induced down-regulated mRNAs (Lewohl et al., 2011; Nunez and Mayfield, 2012), suggesting a role for miRNA-dependent inhibition of gene expression in human alcoholics. Many of the up-regulated miRNAs (miR-15, miR-34, miR-92, miR-140, miR-146, miR-152, miR-194, miR-196, miR-203, miR-369, and let-7) target known modulators of immune signaling, including innate immune toll-like receptors (TLRs) (Nunez and Mayfield, 2012). The miR-146 family was previously shown to be up-regulated by bacterial endotoxin lipopolysaccharides (LPS), which mediate TLR signaling through negative feedback loops involving down-regulation of IL-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) protein levels (Taganov et al., 2006). TLR signaling has been linked to alcohol consumption in human alcoholics and rodent models [for review see (Crews and Vetreno, 2016; Mayfield et al., 2013; Robinson et al., 2014)]. IRAK1 and TRAF6 have also been implicated in mediating alcohol actions (Vallés et al., 2004). These studies support the hypothesis that miRNAs that regulate innate immune signaling are also important in modulating the effects of chronic alcohol in the brain.

In addition to innate immune responses, the differentially expressed miRNAs identified in human frontal cortex from alcoholics coordinate many other biological processes. For example, miR-7 and miR-153 regulate α -synuclein, which plays a role in dopaminergic transmission, activity-dependent maintenance of SNARE complex levels in pre-synaptic terminals (Burgoyne and Morgan, 2011; Burre et al., 2010), and neurodegenerative disorders (Doxakis, 2010). Furthermore, there is a large body of evidence linking α -synuclein accumulation to alcohol dependence in humans and rodents (Bonsch et al., 2005; Taraskina et al., 2008). miR-9 acts as a posttranscriptional regulator of splice variation and neuroadaptations that underlie alcohol tolerance (Farris et al., 2015a; Pietrzykowski et al., 2008). Noteworthy targets of miR-9 include the BK potassium channel *KCNMA1* and *GAD1* which are involved in neuronal excitability and alcohol behavioral phenotypes

(Bettinger and Davies, 2014). Several members of the let-7 family have been reported to regulate mu-opioid, β 2-adrenergic, and dopamine D3 receptors (Chandrasekar and Dreyer, 2009; Pillai et al., 2005). Moreover, miR-101 has been implicated in the modulation of GABAergic transmission in response to alcohol consumption (Saba et al., 2012). Taken together, these studies suggest that alcohol-responsive miRNAs may regulate a variety of neurotransmitter-regulated pathways in the frontal cortex of human alcoholics.

Up-regulated miRNAs in the frontal cortex of alcoholics are also involved in behavioral modifications that occur during the addiction cycle and in other neuropsychiatric disorders. For instance, miR-339 is up-regulated in the frontal cortex of alcoholics and in patients with anxiety disorders (Malan-Muller et al., 2013; Nunez and Mayfield, 2012). Target predicting software proposed adenosine receptor A2a (*ADORA2A*), brain-derived neurotrophic factor (*BDNF*), corticotropin-releasing hormone receptor 2 (*CRHR2*), and sodium-dependent noradrenaline transporter (*SLC6A2*) as possible targets of miR-339 (Malan-Muller et al., 2013). *CRHR2* and *BDNF* have both been implicated in stress disorders and AUD (Moonat and Pandey, 2012; Spence et al., 2009). Thus, miR-339 may be important for anxiety-like behaviors associated with the addiction cycle. miR-152 is up-regulated in the frontal cortex of human alcoholics but down-regulated in depressed individuals (Nunez and Mayfield, 2012; Smalheiser et al., 2014). A predicted target of miR-152 is *ADAM17*, a protein critical for processing of TNF α at the cell surface (Su et al., 2014). The role of TNF α and neuroimmune signaling in AUD suggests a potential neuroimmune function for miR-152. These studies support the overall hypothesis that miRNAs regulate important behavioral aspects of addiction and other psychiatric disorders.

Differentially expressed miRNAs have also been found in the nucleus accumbens of human alcoholics (Mamdani et al., 2015). Although the functional classification of the predicted target genes of the alcohol-responsive miRNAs was similar to those found in the frontal cortex (e.g. cell-adhesion, synaptic transmission, neuronal function), very few of the miRNA hub genes overlapped among brain regions (Lewohl et al., 2011; Mamdani et al., 2015). Only miR-34c-5p was up-regulated in both the cortex and nucleus accumbens (Lewohl et al., 2011; Mamdani et al., 2015). Many of the miRNA targets in the frontal cortex or nucleus accumbens have been implicated in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Minones-Moyano et al., 2011; Saito and Saito, 2012). In the frontal cortex, an inverse relationship was detected between a subset of 27 down-regulated mRNA targets and up-regulated genes involved in Alzheimer's disease (Lewohl et al., 2011). In the nucleus accumbens, down-regulation of the mir-34b/c family was observed (Mamdani et al., 2015). Dysregulation of miR-34b/c triggered downstream transcriptome alterations underlying mitochondrial dysfunction and oxidative stress in Parkinson's disease, which ultimately compromised cell viability (Minones-Moyano et al., 2011). The link between certain miRNA targets and genes implicated in neurodegeneration suggests a neurotoxic effect on cell function in AUD and other diseases.

An additional complexity of miRNA transcriptional regulation is that miRNAs can also regulate other epigenetic factors (Sato et al., 2011). For example, miR-203, which is differentially expressed in human alcoholic brain, targets BMI-1, a component of a polycomb repressor complex 1 (PRC1) that works together with PRC2 to stabilize

epigenetic gene silencing by binding to methylated chromatin (Sato et al., 2011; Wellner et al., 2009). In addition, miR-101 has been shown to specifically repress the expression of a conserved catalytic subunit within PRC2, which is involved in histone methylation and transcriptional silencing (Friedman et al., 2009; Varambally et al., 2008). Other alcohol-responsive miRNAs involved in epigenetic mechanisms include miR-1, which represses histone deacetylase 4 (*HDAC4*) and promotes differentiation of myoblasts (Chen et al., 2006); and miR-152, which targets DNA methyltransferase *DNMT1* (Tsuruta et al., 2011). As discussed earlier, *DNMT1* was down-regulated in the prefrontal cortex, basolateral amygdala, and central nucleus of the amygdala in human alcoholics (Ponomarev et al., 2012). Therefore, it is reasonable to speculate that hypomethylated states could be regulated by alcohol-responsive changes in miR-152 expression. Chronic alcohol-induced changes in the expression of miRNAs could contribute to AUD via epigenetic or other cellular functions.

3.3: Long non-coding RNAs as transcriptional regulators

Long non-coding RNAs (lncRNAs) also regulate transcription of RNA and may be important in AUD. lncRNAs represent the most abundant class of ncRNAs in brain (Jia et al., 2010), and compared to the approximately 20,000 protein-coding genes in humans (Carninci and Hayashizaki, 2007; Jia et al., 2010; Ravasi et al., 2006), annotations indicate that there are > 16,000 lncRNAs, the majority of which show tissue- and temporal-specific expression in the CNS (Derrien et al., 2012). lncRNAs are critical for normal cellular function, including the regulation of gene expression (Wang and Chang, 2011), cell proliferation and differentiation (Guttman et al., 2011), as well as the pathophysiology of disease (Wapinski and Chang, 2011). Similar to protein-coding transcripts, localization of individual lncRNAs is brain-region specific (Belgard et al., 2011; Mercer et al., 2008). The expression of lncRNAs was shown to be dynamically regulated in human alcoholic brain (Farris et al., 2015a). In the prefrontal cortex of human alcoholics, an alcohol-responsive gene module contained multiple lncRNAs, such as *NCRNA00092*, *NCRNA00174*, and *NCRNA00284* (Farris et al., 2015a; Farris and Mayfield, 2014), suggesting a potential role for lncRNAs in AUD.

A continual challenge for gene expression profiling is that comparing expression levels of individual genes between alcoholic cases and controls may not be sufficiently powered to detect significant differences or determine causal candidate genes. Therefore, many researchers couple GWAS with expression quantitative loci (eQTL). An individual locus can account for a fraction of the genetic variance of a gene expression phenotype (Nica and Dermitzakis, 2013). Standard eQTL analysis involves a direct association test between markers of genetic variation and gene expression levels typically measured in hundreds of individuals (Nica and Dermitzakis, 2013). Genetic markers that are simultaneously associated with disease states are a powerful tool. For example, if one allele is more frequent in alcoholic cases than controls and is causal for gene expression effects of a nearby gene, that is known to be important for AUD, then it is probable that causality can be established (Nica and Dermitzakis, 2013). Among the enriched eQTL sets in the nucleus accumbens of human alcoholics, the most significant GWAS signals included the lncRNA *PKI55* (Mamdani et al., 2015). This suggests a causal relationship between lncRNA expression and

AUD, further establishing a role for these ncRNAs in the regulation of gene expression and disease states such as AUD.

Although the role of lncRNAs in alcohol dependence is still unclear, they are known to 1) regulate epigenetic factors and gene expression (Khalil et al., 2009; Lee, 2012; Wang et al., 2011), 2) act as endogenous competitors (Cesana et al., 2011), 3) regulate alternative splicing events (Barry et al., 2014; Massone et al., 2011; Tripathi et al., 2010), and 4) alter synaptic plasticity (Bond et al., 2009), all of which are important mechanisms in AUD. Interestingly, there may be overlap in drug-responsive lncRNAs that are altered by alcohol dependence and other substance abuse disorders. For example, nuclear-enriched abundant transcript 2 (*NEAT2*), a lncRNA important in synaptogenesis (Bernard et al., 2010), was up-regulated in human alcoholic brain (Kryger et al., 2012). *NEAT2*, and other related lncRNAs, were also up-regulated in the nucleus accumbens of heroin (e.g., *NEAT1*, *MIAT*, and *MEG3*) and cocaine abusers (e.g., *MIAT*, *MEG3*, and *EMX2OS*) (Albertson et al., 2004; Michelhaugh et al., 2011). Thus, different types of substance abuse/dependence may produce overlapping changes in related lncRNAs. Given that lncRNAs fulfill diverse roles in the CNS, even small changes in lncRNA expression could significantly impact patterns of chronic alcohol consumption (Fig. 1).

4. Functional systems associated with alcohol dependence

In addition to discrete mechanisms of transcriptional regulation, analysis of the human transcriptome has identified alterations in ion channel subunits and other systems that may be associated with alcohol dependence. In the following sections, we discuss several of these gene categories. Jones et al. (2015) reviews other candidate alcohol-responsive genes, including variants of the following: the mu-opioid receptor (*OPRM1*), dopamine transporter (*SLC6A3*), serotonin transporter (*SLC6A4*), dopamine receptor D2 (*DRD2*), and the $\alpha 3$ subunit of the glycine receptor (*GLRA3*).

4.1: GABA

Alcohol potentiates GABA_A receptor-mediated responses and enhances inhibitory neurotransmission. Alterations in GABAergic signaling have been hypothesized to underlie all three stages of addiction. The rewarding properties of alcohol were hypothesized to depend on GABA_A signaling in the nucleus accumbens and amygdala (Koob and Volkow, 2010; Volkow et al., 2016). Blockade of dopamine and GABA_A receptors in the ventral pallidum prevents the reinforcing effects of alcohol (Koob and Volkow, 2010; Volkow et al., 2016). GABAergic signaling has also been implicated in increasing withdrawal-induced anxiety (Leung et al., 2015; Smith et al., 1998). Furthermore, neurocircuitry changes associated with drug- and cue-induced reinstatement after extinction have been linked to GABAergic projections from the nucleus accumbens to the ventral pallidum (Kalivas and O'Brien, 2008).

Some of the top candidate genes implicated in alcohol consumption in humans are transcripts for specific GABA receptor subunits (Trudell et al., 2014). An RNA-seq study compared GABAergic gene expression in human postmortem hippocampus (Enoch et al., 2012). Alcoholics had lower expression of multiple genes associated with GABA receptors;

these included specific subunits for GABA_A and GABA_B receptors (i.e. *GABBR1*, *GABRA2*, *GABRG1*, *GABRG2*). Similar findings were reported in a separate RNA-seq study of human postmortem hippocampus (Zhou et al., 2011). The differential expression of GABA receptor subunits in human alcoholics has also been observed in the prefrontal cortex. For example, *GABRA3*, *GABBR1*, and *GABBR2* were primary network contributors in human prefrontal cortex that were associated with lifetime alcohol consumption (Farris and Mayfield, 2014). A meta-analysis of candidate gene association studies also identified a strong association of the GABA_A receptor subunit $\alpha 2$ (*GABRA2*) with alcohol dependence along with evidence for $\alpha 6$ (*GABRA6*) and $\gamma 2$ (*GABRG2*) subunits (Li et al., 2014). Enoch and colleagues review earlier studies that nominated *GABRA2* (Enoch et al. (2009)).

Alcohol alters the expression of genes involved in neurotransmission, specifically those implicated in GABA synthesis and transport across the synaptic cleft. For example, *GAD1*, *SLC6A1*, *PRAF2*, and *GPHN* were down-regulated in the hippocampus of human alcoholics as well as in cocaine addicts (Enoch et al., 2013; Enoch et al., 2012; Zhou et al., 2011). *GAD1* encodes GAD67, the rate-limiting enzyme for the synthesis of GABA (Asada et al., 1996); *SLC6A1* encodes the neuronal GABA transporter GAT-1; *PRAF2* encodes the protein implicated in the feedback regulation of synaptic transmission (Koomoa et al., 2008); and *GPHN* encodes gephyrin, a scaffolding protein that anchors GABA_A receptors to the postsynaptic skeleton. These findings provide evidence for a coordinated dysregulation of GABA receptor synthesis and transport that could affect synaptic transmission in human alcoholics.

4.2: Glutamate

Ionotropic and metabotropic glutamate receptors are also well established targets for alcohol action (D'Souza, 2015). Glutamate, which modulates dopamine cells and dopamine release in the nucleus accumbens, is critical for the rewarding effects of drugs of abuse and contributes to the neuroplastic changes associated with conditioned responses and craving (Koob and Volkow, 2010; Volkow et al., 2016).

The glutamate ionotropic receptor NMDA Type Subunit 2B (*GRIN2B*) and the glutamate ionotropic receptor AMPA Type Subunit 1 (*GRIA1*) were identified as hub genes in transcriptome analysis of human alcoholic prefrontal cortex (Farris and Mayfield, 2014). *GRIN2B* was up-regulated in the hippocampus of human alcoholics (Zhou et al., 2011). Analysis of frontal cortex modules also revealed up-regulated genes involved in synaptic transmission in alcoholics, particularly at glutamatergic synapses (Ponomarev et al., 2012). Genes from this alcohol-responsive module included *GRIN1*, dynamin (*DNMI*), syntaxin (*STX1A*), synapsin 1 (*SYN1*), synaptophysin (*SYP*), and the vesicular glutamate transporter 1 (*VGLUT1*, *SLC17A7*). The GC content of the glutamatergic genes was greater than average, suggesting coordinated synaptic up-regulation in alcohol abusers (Ponomarev et al., 2012). Two additional up-regulated genes from this module, *GIPC1* and *MIB2*, have roles in glutamate transmission and are respectively involved in NMDA receptor trafficking and ubiquitination of the NMDA NR2B subunit (Jurd et al., 2008; Yi et al., 2007). Up-regulation of NR2B-NMDA receptor activity within the dorsomedial striatum by alcohol contributed to

synaptic dysregulation and excessive alcohol intake and relapse drinking in rats (Wang et al., 2010), suggesting overlapping dysregulation of glutamatergic signaling in different species. Coupled with down-regulation of GABA receptors and genes involved in GABA synthesis/transport, the increased glutamatergic signaling suggests an excitotoxic shift in synaptic signaling in the alcoholic brain.

4.3: Voltage-gated sodium channels

The voltage-gated sodium channel is another putative quantitative trait gene for alcohol drinking behavior (Davies et al., 2003; Mulligan et al., 2006). In the prefrontal cortex of human alcoholics, three high confidence transcripts for the voltage-gated sodium channel (*SCN4B*, *SYT13*, and *TBC1D9*) correlated with lifetime alcohol consumption (Farris and Mayfield, 2014). In neurons, *SCN4B* can override the effects of other beta subunits (Yu et al., 2003), modify sodium channel firing rates (Grieco et al., 2005), and structurally alter neuronal projections (Oyama et al., 2006). There are three coding isoforms of *SCN4B* in humans, but only the shortest coding variant (NM_001142349) was significantly correlated with alcohol consumption (Farris and Mayfield, 2014). WGCNA identified this variant as a phenotypic focal point within the network that was distinct from the other two coding isoforms of *SCN4B* (Farris et al 2014). NM_001142349 was positively co-expressed with another splicing variant, *SCN8A*, a sodium channel alpha subunit expressed within neuronal synapses (Farris and Mayfield, 2014; Schaller and Caldwell, 2000). Although the positive co-expression of *SCN4B* and *SCN8A* splice variants may signify their functional importance, this is difficult to predict because there have been few large-scale studies on the role of alternative splicing in alcohol consumption (Lee et al., 2014).

4.4: Enzymes and alcohol metabolism

Oxidative metabolism of alcohol occurs mainly in the liver via a major pathway in which the cytosolic enzyme alcohol dehydrogenase (ADH) produces a highly reactive intermediate, acetaldehyde. In addition to ADH, a group of enzymes, known as cytochrome P450 isozymes (including CYP2E1, 1A2, and 3A4), contribute to the oxidation of alcohol to acetaldehyde in the liver. Acetaldehyde is subsequently metabolized by acetaldehyde dehydrogenase (ALDH) to form acetate, which is eliminated from the body. A catalase-mediated reaction in the peroxisomes is considered a minor pathway for alcohol metabolism in the periphery. In the brain, however, alcohol is metabolized primarily through the activity of catalase. Other metabolic pathways, such as mitochondrial cytochrome P450, also produce acetaldehyde locally within the brain following alcohol consumption (Zakhari, 2013). Acetaldehyde formation mediates the behavioral and neurobiological effects of alcohol, but its role is highly dependent on dose and location (e.g. periphery or brain). For instance, studies have reported that intracranial ventricular administration of acetaldehyde increases ethanol consumption and preference in rodents, while peripheral administration of higher doses of acetaldehyde produces a conditioned taste aversion in several rat lines (reviewed by (Quertemont et al., 2005)).

Genetic polymorphisms are associated with risk for AUD, as well as intermediate phenotypes at the molecular and behavioral level (e.g. *ADH*, *ALDH*, *COMT*, *MAOB*, *HTR2B*, *FBKP5*) (Ducci and Goldman, 2012). *ADH* and *ALDH* variants are some of the

strongest genetic predictors of risk for developing AUD. *ADH* and *ALDH* are typically associated with decreased risk for alcohol dependence since variations result in increased toxic effects of acetaldehyde in the periphery (Edenberg and Foroud, 2006). Among the 39 differentially expressed genes identified in prefrontal cortex of human alcoholic subjects, one was from the *ADH* gene family, six were from the *ALDH* family, and four were from the cytochrome P450 family (Zhang et al., 2014a), suggesting coordinated disruption in alcohol metabolism. Also, an evolutionarily conserved GABA synthesis pathway in dopamine neurons is mediated by the aldehyde dehydrogenase, *ALDH1A1*, rather than the conventional enzymes, *GAD65* and *GAD67* (Kim et al., 2015). In midbrain dopamine neurons, GABA co-release is modulated by ethanol at blood alcohol concentrations observed during binge drinking, and decreased *ALDH1A1* leads to enhanced alcohol intake and preference (Kim et al., 2015). Taken together, this implies that alcohol metabolism in brain is involved in reward-based behavior and addiction.

Mitochondria also participate in alcohol metabolism by converting acetaldehyde into acetate (Manzo-Avalos and Saavedra-Molina, 2010). An RNA-seq study found 56 genes involved in mitochondrial function that were differentially expressed in postmortem frontal cortex of alcoholic subjects (Zhang et al., 2014a). Additionally, previous microarray studies of human alcoholic brain tissue have shown differential expression of genes related to mitochondrial function (Liu et al., 2006). Therefore, it can be hypothesized that the differential expression of genes related to alcohol metabolism (e.g. *ADH*, *ALDH*, *P450*) in brain could alter the reinforcing effects of alcohol and change drinking behavior. This hypothesis was supported by injecting lentiviral vectors (anti-catalase shRNA or rADH1), which inhibit catalase synthesis or increase activity of ADH, respectively (Karanian et al., 2011). Administration of the anti-catalase lentiviral vector into the ventral tegmental area significantly reduced ethanol consumption and ethanol-stimulated dopamine release in the nucleus accumbens shell; whereas, the rADH1-encoding vector facilitated an increase in ethanol intake (Karanian et al., 2011). This study reinforces the key role of metabolism in chronic alcohol consumption and demonstrates how network-wide changes in differential expression of genes important in alcohol metabolism can significantly alter drinking behavior.

4.5: Immune/inflammatory and stress signaling

Microarray studies have consistently highlighted a role for immune-related genes in the human alcoholic brain (Lewohl et al., 2000; Liu et al., 2006; Mayfield et al., 2002). The genomic studies provided the initial evidence that immune/inflammatory genes may be important in excessive alcohol consumption. Behavioral evidence subsequently validated the role of some of these genes in ethanol drinking in mouse models (Mayfield et al., 2016). Ethanol treatment *in vivo* has been shown to increase the expression of TLRs, resulting in NF- κ B activation and cytokine induction (Crews et al., 2013; Lippai et al., 2013). IKK β regulates activation of NF- κ B, and both pharmacologic inhibition and genetic deletion of IKK β in the nucleus accumbens and central nucleus of the amygdala reduced voluntary alcohol drinking in mice (Truitt et al., 2016), providing further support for NF- κ B signaling in chronic drinking. Neurodegeneration caused by chronic alcohol exposure is also partially due to signaling through the TLR and NF- κ B signaling pathways (Alfonso-Loeches et al., 2010; Qin et al., 2007). Chronic exposure to alcohol alters the expression of other immune-

related genes that regulate TLR signaling, such as *HMGB1* (a damage associated molecular pattern that can activate multiple TLRs) (Crews et al., 2013).

Microarray analysis of human postmortem hippocampal tissue revealed large fold-changes in genes associated with interleukin receptor signaling, in particular, up-regulated IL-1 and IL-6 receptors (McClintick et al., 2013). A cascade of events downstream of the IL-1 receptor complex results in the activation of important signaling proteins, such as mitogen-activated kinases (JNK, p38, ERK1/2), as well as transcription factors, including NF- κ B (p65 and p50 subunits) and c-Jun (a subunit of AP-1), which control expression of inflammatory and catabolic genes. In contrast, IL-6 receptor signaling occurs primarily via JAK/STAT, resulting in both pro- and anti-inflammatory gene expression. In mice with a genetic predisposition to high alcohol consumption, IL-1 and IL-6 receptor signaling was up-regulated (Mulligan et al., 2006). Differential expression of IL receptors (e.g., IL-6), NF- κ B signaling, and chemokine receptors/ligands have also been observed in the hippocampus of cocaine- and alcohol-addicted individuals (Zhou et al., 2011). In addition, bilateral infusions of IL-10 into the basolateral amygdala reduced binge drinking in mice (Marshall et al., 2016). Differential expression of the JAK/STAT pathway was observed in the nucleus accumbens (Mamdani et al., 2015). Alterations in JAK/STAT signaling were also found in rhesus macaques chronically exposed to ethanol (Asquith et al., 2014; Sureshchandra et al., 2016). Thus, in addition to changes in canonical TLR signaling, chronic ethanol exposure alters many other immune signaling pathways (i.e. IL receptors, JAK/STAT). The effects of alcohol on neuroimmune systems have been reviewed recently by Crews and Ventreno (2016) and Montesinos et al. (2016). Overlapping changes in immune signaling categories have been found among different brain regions (Liu et al., 2006; McClintick et al., 2013; Zhou et al., 2011). For example, general chemokine and interleukin responses were observed in the frontal cortex, motor cortex, hippocampus, and nucleus accumbens of human alcoholics (Liu et al., 2004; Mamdani et al., 2015; Mayfield et al., 2002; McClintick et al., 2013). The brain-regional regulation of immune/inflammatory pathways by alcohol is not well established and represents a current area of focus for addiction researchers. The role of different populations of brain cells is another emerging area of study for drugs of abuse and addiction behaviors (Lacagina et al., 2017) and is also discussed later in this review.

Genes involved in cellular stress responses are also sensitive to alcohol exposure. The glucocorticoid receptor (NR3C1) was down-regulated in hippocampus from alcoholics, whereas negative regulators of the pathway (FK506 binding proteins) were increased over two-fold (McClintick et al., 2013). The *TXNIP* gene encoding thioredoxin-binding protein, which can be transcriptionally induced by glucocorticoids, was increased in alcoholics. *TXNIP* acts as a major regulator of cellular redox signaling and protects cells from oxidative stress, suggesting an enhanced cellular stress response induced by chronic alcohol exposure. Moreover, alcohol abuse, chronic drinking and early stages of withdrawal increased the expression of genes involved in behavioral stress responses, such as cortisol-releasing-hormone (*CRH*), which can activate glucocorticoid receptors and enhance expression of downstream inflammatory genes (e.g. *IL1B*, *TGFB1*) (Armario, 2010). Cortisol is known to modify learning and memory (Stephens and Wand, 2012). Exposure to high levels of behavioral stress and increased cortisol can transiently block memory retrieval (van Stegeren, 2009), with retrieval of emotional memory more strongly affected than that of

neutral memory. Potential effects on emotionally charged memory and habit learning may be particularly relevant in the addictive process. Furthermore, both behavioral stress and alcohol exposure can enhance glucocorticoid-induced priming of inflammatory responses (Ellis, 1966; Frank et al., 2010). Behavioral stress and alcohol exposure also mediate inflammation via other common mechanisms. For example, stress increases gut/intestinal permeability and allows toxins to leak from the gut lumen (Garate et al., 2013). Alcohol also contributes to “leaky gut,” allowing bacterial toxins to enter the bloodstream (Adachi et al., 1995), which activates innate immune and inflammatory signaling via peripheral and central processes (Mayfield et al., 2013). Exposure to alcohol and stress may alter similar genes and inflammatory pathways that collectively contribute to the development and/or progression of AUD. Therefore, stress-induced signaling (both on the cellular and behavioral level) could play a key role in the negative affect component of withdrawal. Neuroplastic changes in CRH and glucocorticoid receptors could also participate in the enhanced sensitivity to stressors that drive relapse behavior (Koob and Volkow, 2010; Volkow et al., 2016).

5. Cell-type specific effects of alcohol in human brain tissue

Understanding brain function in disease states requires detailed study of how specific populations of cells interact in a dynamic environment. Different cell types use distinct transcriptional mechanisms that result in remarkable heterogeneity of cellular transcriptomes (Doyle et al., 2008; Sugino et al., 2006). Recently, an Alzheimer’s disease model showed that transcriptome changes observed in whole tissue are driven primarily by specific cell types and not necessarily by global transcriptional regulation (Srinivasan et al., 2016).

As mentioned in the preceding section, alcohol may differentially target cell types across brain regions. Analysis of human frontal cortex, central nucleus of the amygdala, and basolateral amygdala revealed discrete effects of alcohol on different cell types in these regions (Ponomarev et al., 2012). The basolateral amygdala showed the most changes in cell-type composition, and the frontal cortex showed the fewest. The neuronal and microglial distributions in the amygdalar regions were inversely shifted, suggesting a decrease in the number of neurons and an increase in microglia. Similarly, analysis of the nucleus accumbens and hippocampus revealed that alcohol differentially targets cell types in these regions (Mamdani et al., 2015; McClintick et al., 2013). Neuronal expression modules were negatively correlated with lifetime alcohol consumption, while astrocytic and microglial expression modules were positively correlated. Again, this suggested a loss of neuronal function in tandem with astrocytic and microglia activation in brain tissue from human alcoholics. This is consistent with literature showing a general degradation of neurons as well as activation and proliferation of microglia after chronic alcohol exposure (Crews et al., 2011), which may contribute to addiction-related neuroplastic changes. Oligodendrocytes also show differential gene expression in multiple brain regions after chronic alcohol exposure. In the hippocampus, 83% of the differentially expressed transcripts enriched in oligodendrocytes were expressed at lower levels in alcoholics (McClintick et al., 2013). In the human frontal cortex, several myelin-related genes (*MOBP*, *OPALIN*, *UGT8*, and *ERMN*) were alcohol-responsive (Farris et al., 2015a). Abnormal white matter expression plays an essential role in brain morphology and behavioral responses to alcohol and other drugs of abuse (Pfefferbaum et al., 1997; Sokolov, 2007). The

discrete alcohol-induced changes in cellular transcriptomes indicate that cell types may be important targets for future drug development.

6. Cell-type specific transcriptome techniques

Brain-region specific gene expression analysis from human alcoholic versus control subjects has allowed unprecedented access to gene co-expression networks that are altered by chronic alcohol consumption. However, the cellular heterogeneity of individual brain regions could compromise interpretations of gene expression data. First and foremost, tissue heterogeneity could restrict or mask detection of significant expression changes occurring within a particular cell type, especially if this population represents a smaller proportion of the total cells in the sample. Second, *in silico* tools that are currently used in cell-type specific analyses limit interpretations because an observed change in gene expression could be due to the gain or loss of certain cell types, an expression change in all cells or in a subset of cells, or combinations of all of these conditions.

Tools are available for cell-type specific transcriptome analysis in rodents, including isolation of cell types for culture and isolation of live cell types using FACS sorting or antibody-specific beads. Isolations of mouse brain cell types (e.g. astrocytes, microglia, endothelial cells) have been used to create databases of cell-type specific transcriptomes in several brain regions. Bioinformatics analyses of cell types have identified thousands of cell-type enriched genes and splicing isoforms (Zhang et al., 2014b). These databases can be applied to existing and future datasets to identify novel markers for cell identification, tools for genetic manipulation, and insights into disease states. FACS sorting has similarly been used to create databases of cell-type specific transcriptomes. For example, a novel Tmem119 antibody and FACS-sorted microglia RNA-seq revealed the expression of novel ligands, receptors, and disease-associated genes. This strongly suggested new microglial functions in immune effector functions, regulation of catecholamines, and synapse formation (Bennett et al., 2016). FACS sorting was also used to determine cell-type specific changes after LPS administration, revealing that LPS-altered genes in certain cell types were not found in whole tissue preparations (Srinivasan et al., 2016). Moreover, the overall direction of change in gene expression was also cell-type specific (e.g. down-regulated in microglia but up-regulated in astrocytes) (Srinivasan et al., 2016).

FACS sorting and antibody-specific beads have been utilized to isolate live human neurons as well as single cell nuclear transcriptomes in postmortem human brain (Krishnaswami et al., 2016). More commonly, cultured human cells have been sequenced to identify cell-type specific transcriptomes (Srinivasan et al., 2016; Zhang et al., 2016). However this does not allow for analysis of brain regional differences in individual cell types. Thus, single cell transcriptome analysis in human brain is preferable for this purpose (Darmanis et al., 2015). Similar to mouse cell-type transcriptome databases, these methods provide novel markers for cell identification, tools for genetic manipulation, and insights into disease states.

Development of more sophisticated algorithms and analyses in the human brain may improve the ability to uncover cell-specific gene markers that mediate disease states (Xu et al., 2013). Just as distinct brain regional circuits are associated with different phases of

addiction, cell-specific signaling among brain regions may also be critical in the addiction process. Developing tools to isolate and sequence live cell types from different human brain regions will be a key step in defining the alcoholic transcriptome on an unprecedented level. Cell-type specific changes in particular genes and/or cell-type differences in the direction of gene expression (Fig. 2) may eventually be used to develop drugs that target individual cell types (e.g. reactive astrocytes, proinflammatory microglia).

7. Summary and future directions

RNA molecules exist as orchestrated networks throughout the genome, and are regulated, in part, by genetic variants, alternative splicing, and epigenetic modifications. Transcriptome approaches are continually being refined, improving our understanding of the molecular etiology and progression of AUD. We can now investigate how alcohol changes DNA regulation via epigenetic modifications, or how it alters transcriptional regulation via miRNAs and lncRNAs, or even how gene expression changes in individual cell types potentially mediate the transition from alcohol use to the “dark side of addiction” (Koob and Volkow, 2010; Volkow et al., 2016) (Fig. 3). Sequence data can be integrated with protein data, physiological function, phenotypic traits, and other factors such as the microbiome, to create a more cohesive picture of the disease state (Farris and Mayfield, 2014). This information, coupled with corresponding information from animal models (Truitt et al, 2016), aims to uncover improved treatment strategies for AUD (Warden et al., 2016), and may one day be used to target an individual’s transcriptome profile. Advances in “precision” and “personalized” medicine could lead to improved therapies that surpass those obtained by current *in silico* bioinformatics approaches (Shin et al., 2016; Sliwczynski and Orlewska, 2016).

Traditional gene expression techniques (e.g. microarray analyses) have already uncovered functional categories of genes, such as splice variants of ion channels or genes involved in innate immune/inflammatory responses, that may contribute to alcohol dependence in humans. Microarrays produce long lists of individual candidate genes, but have not shed light on how these genes might interact to produce an alcohol-dependent phenotype. Analyzing gene co-expression networks from alcoholic brain tissue generates a more integrated, systems-level view, spanning multiple cell types or brain regions (Fig. 3). Gene networks have also highlighted a novel role for transcriptional regulation in development of alcohol dependence via epigenetic mechanisms and non-coding RNAs (Fig. 1). Bioinformatics analyses and sequencing techniques, such as RNA-seq, now permit unparalleled insight into the complexity of the transcriptome. Although still in the early stages of use, RNA-seq has been used to determine spatial organization of the transcriptome (Lee et al., 2014) and extract some of the subtle expression differences induced by individual neurons and their microenvironments (Lovatt et al., 2014). This and other rapidly advancing computational approaches promise to improve discovery of the functionally relevant molecules and pathways involved in AUD and lead to more targeted treatment approaches, with the potential to integrate changes in gene networks, cell types, and brain regions in the different stages of addiction.

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Highlights

- Overall, an integrated perspective on alcohol-responsive transcriptional changes in the human brain underlying the regulatory gene networks that contribute to alcohol dependence is outlined.
- Human alcoholic transcriptome analysis in postmortem tissue requires high quality from established brain banks such as the New South Wales Tissue Resource Centre at The University of Sydney, Australia.
- Alcohol alters DNA methylation and expression of individual genes.
- Distinct noncoding RNA regulatory mechanisms in brain are thought to influence the development and progression of psychiatric disorders.

Figure 1.

A hypothetical diagram for the role of transcriptional regulation in AUD. Chronic alcohol consumption can alter regulation of transcription via epigenetic modifications, including miRNAs and lncRNAs. Alterations in these regulatory mechanisms result in aberrant downstream gene expression changes in functional systems known to contribute to alcohol dependence (e.g. neuronal transmission, ion channels, immune signaling, stress response).

Figure 2.

Total tissue versus cell-type specific analyses. Left Panel: Example volcano plot from an analysis of a cell-type specific isolation. The wider distribution of the plot signifies a greater number of statistically significant differentially expressed genes associated with chronic alcohol exposure. Right Panel: Example volcano plot from an analysis of a total tissue preparation. Note the smaller distribution and fewer statistically significant differentially expressed genes. Example data source: (<https://raw.githubusercontent.com/brennanpincardiff/RforBiochemists/master/data/microArrayData.tsv>”).

Figure 3.

RNA-seq systems approach for understanding alcohol dependence. Sequencing can be used to evaluate changes in regulation of DNA and RNA expression in human alcoholic and control cases. Information from sequencing can then be superimposed with other variables such as protein data, physiological function, or other phenotypic information to identify mechanisms and potential treatment options for AUD. Adapted from (Farris and Mayfield, 2014).

Table 1

Summary of Human Alcoholic Gene Expression Profiling Studies

Functional Category	Authors	Brain Regions Profiled	Technique	Brain Bank
Epigenetic Regulation	Farris et al., 2015b	Hippocampus	RNA-seq/Chip-seq	University of Miami
	Ponomarev et al., 2012	Superior frontal cortex, basolateral and central amygdala	Microarray	New South Wales
miRNA-regulation	Zhou et al., 2011	Hippocampus	RNA-seq/Chip-seq	University of Miami
	Farris et al., 2015a	Superior frontal gyrus	RNA-seq	New South Wales
	Farris et al., 2015b	Hippocampus	RNA-seq/Chip-seq	University of Miami
	Lewohl et al., 2011	Frontal cortex	miRNA array	University of Queensland
	Mamdani et al., 2015	Nucleus accumbens	Microarray	New South Wales
Long non-coding RNAs	Farris & Mayfield, 2014	Frontal cortex	RNA-seq	New South Wales
Ion Channels & Signal Transduction	Enoch et al., 2012	Hippocampus	RNA-seq	University of Miami
	Enoch et al., 2013	Hippocampus	RNA-seq	University of Miami
	Farris et al., 2015a	Superior frontal gyrus	RNA-seq	New South Wales
	Ponomarev et al., 2012	Superior frontal cortex, basolateral and central amygdala	Microarray	New South Wales
	Zhou et al., 2011	Hippocampus	RNA-seq/Chip-seq	University of Miami Brain Bank
Immune & Stress Response	Mamdani et al., 2015	Nucleus accumbens	Microarray	New South Wales
	McClintick et al., 2013	Hippocampus	Microarray	New South Wales
Metabolism	Zhang et al., 2014a	Basolateral prefrontal cortex	Microarray	New South Wales